Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: Implications for oxygen sensing and hypoxic signaling in eukaryotes

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Summary

Eukaryotic cells respond to low-oxygen concentrations by upregulating hypoxic nuclear genes (hypoxic signaling). Although it has been shown previously that the mitochondrial respiratory chain is required for hypoxic signaling, its underlying role in this process has been unclear. Here, we find that yeast and rat liver mitochondria produce nitric oxide (NO) at dissolved oxygen concentrations below 20 μM. This NO production is nitrite (NO_2^-) dependent, requires an electron donor, and is carried out by cytochrome *c* oxidase in a pH-dependent fashion. Mitochondrial NO production in yeast is influenced by the YHb flavohemoglobin NO oxidoreductase, stimulates expression of the hypoxic nuclear gene *CYC7*, and is accompanied by an increase in protein tyrosine nitration. These findings demonstrate an alternative role for the mitochondrial respiratory chain under hypoxic or anoxic conditions and suggest that mitochondrially produced NO is involved in hypoxic signaling, possibly via a pathway that involves protein tyrosine nitration.

Introduction

Several previous studies have implicated the respiratory chain in hypoxic signaling in eukaryotes (Kwast et al., 1999; Poyton, 1999; Poyton et al., 2003; Chandel et al., 1998). It has been proposed that the respiratory chain produces increased levels of reactive oxygen species (ROS) when cells experience reduced oxygen levels and that these ROS are involved in hypoxic signaling. Support for this mitochondrial model for oxygen sensing comes from studies with both yeast and mammalian cells. Studies with yeast have demonstrated that exposure to anoxia or hypoxia leads to a transient increase in mitochondrially generated oxidative stress, enhanced levels of protein and DNA oxidation (Dirmeier et al., 2002), and increased expression of *SOD1* resulting from increased superoxide levels. And studies with mammalian cells have implicated ROS in stabilization of HIF-1α, an important regulator of hypoxic genes (c.f., Kaelin, 2005).

Although yeast and mammalian cells experience transient oxidative stress upon exposure to hypoxia, the relationship between this and hypoxic signaling is still poorly understood. On the one hand, support for the involvement of oxidative stress in hypoxic signaling in yeast comes from the finding that some genes involved in the oxidative stress response are induced by anoxia (Lai et al., 2005; ter Linde et al., 1999). On the other hand, hypoxic yeast genes are not induced by exogenously added oxidants (Causton et al., 2001; Gasch et al., 2000), suggesting that ROS alone are not sufficient for hypoxic signaling and that the mitochondrial respiratory chain may influence hypoxic signaling via other mechanisms.

An important hint concerning an additional role for the mitochondrial respiratory chain in hypoxic signaling comes from recent studies on the yeast flavohemoglobin YHb, a homolog of mammalian cytohemoglobins. YHb is a NO oxidoreductase which plays an essential role in both oxidative and nitrosative stress responses (Liu et al., 2000; Zhao et al., 1996) and which, as such, regulates levels of peroxynitrite ($ONOO^-$), a substrate for protein tyrosine nitration (Radi, 2004). YHb is present in the cytosol and mitochondrial matrix of normoxic cells but resides exclusively in the promitochondrial matrix in anoxic cells (Cassanova et al., 2005). The presence of YHb exclusively in the promitochondria of anoxic cells is surprising considering that these cells are grown in the absence of oxygen and have greatly reduced levels of oxidative stress (Dirmeier et al., 2002). Because YHb functions to consume NO, this observation implies that NO is produced in anoxic yeast promitochondria. These findings, together with earlier reports that promitochondria retain low levels of mitochondrial respiratory proteins (Dagsgaard et al., 2001) and the ability to respire (David and Poyton, 2005), suggest that the mitochondrial respiratory chain functions with an alternative electron acceptor at low-oxygen concentrations and that the product of this chain is NO. This hypothesis is supported by mammalian cell studies which have demonstrated that mitochondria produce elevated levels of NO under hypoxia (Valdez et al., 2004; Schild et al., 2003) and that some of this NO synthesis is not catalyzed by NO synthase (NOS) (Lepore et al., 1999; Chen et al., 2002; Agvald et al., 2002).

In this study, we examined the involvement of mitochondria in NO production and hypoxic signaling. We have found that both yeast and rat liver mitochondria are capable of producing NO from nitrite (NO_2^-) when exposed to low-oxygen concentrations. This reaction is dependent on the respiratory chain. We have also identified cytochrome *c* oxidase as the mitochondrial enzyme that functions to reduce NO_2^- to NO. In addition, we have been able to demonstrate that yeast cells experience a transient increase in protein tyrosine nitration when exposed to anoxia and that mitochondrially generated NO can be linked to the induction of *CYC7*, a hypoxic yeast gene.

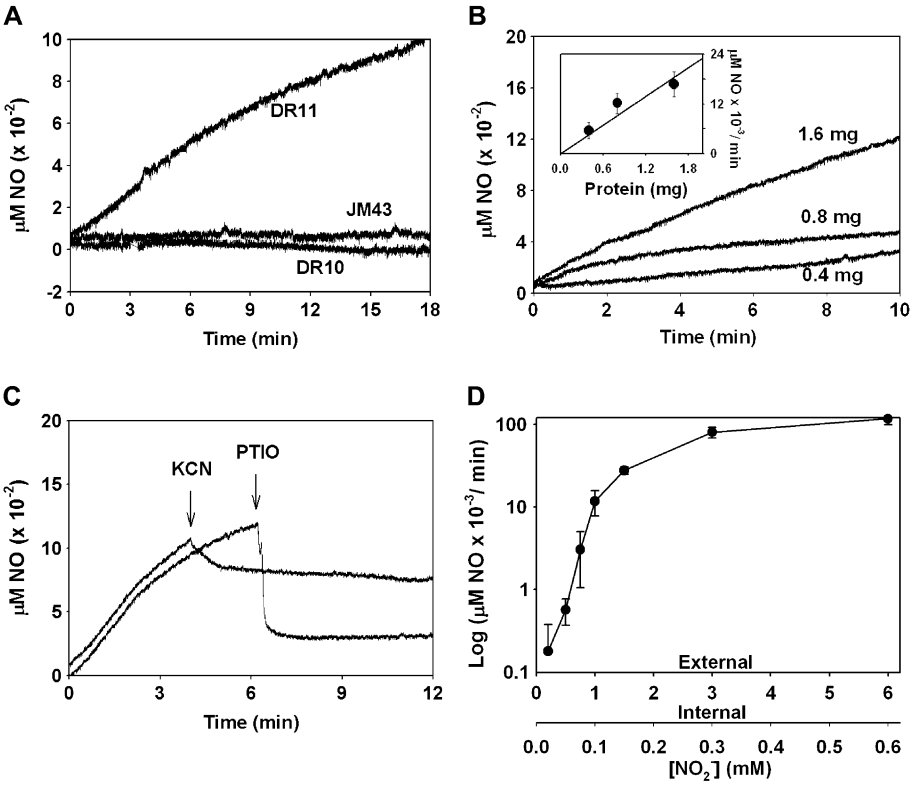

Figure 1. NO production by yeast

A) NO production by isolated yeast mitochondria. Isolated mitochondria (400 μg protein/ml) from strains JM43, DR11, and DR10 were preincubated for 12 min at 28°C in NO Assay Medium. Then, $NaNO_2$ was added to a final concentration of 1 mM and NO production recorded with a NO electrode.

B) Dose-dependent NO production in isolated DR11 mitochondria. Different amounts of isolated mitochondrial protein were analyzed for their ability to produce NO following the protocol described above in (**A**). *Inset*, Initial rate of NO production versus mg mitochondrial protein.

C) Effects of the NO scavenger PTIO and KCN on NO production by isolated yeast mitochondria. NO production from DR11 yeast mitochondria (400 μg protein/ml) was measured as described in (**A**). At the times indicated by arrows, KCN or PTIO were added to final concentrations of 1 mM and 10 mM, respectively.

D) Effect of the NO_2^- concentration on the NO production by isolated yeast mitochondria. NO production from 400 μg mitochondrial protein/ml was measured as described in (**A**) in the presence of different concentrations of added NO_2^- (the external concentration). The internal concentration of NO_2^- was measured as described in Experimental Procedures.

Results

NO production in yeast

To determine if yeast cells are capable of endogenous NO synthesis we assayed three isogenic yeast strains: JM43, which is *YHB1*$^+$ and ρ^+ (respiration-proficient); DR11, which is *yhb1*$^-$ and ρ^+; and DR10, which is *yhb1*$^-$ and ρ^o (respiration-deficient) (Zhao et al., 1996). From Figure S1 it is clear that yeast cells are capable of NO production and that the rate of NO production by DR11 cells is substantially higher than the rates in NO production in JM43 or DR10 cells (Figure S1). These findings indicate that YHb suppresses endogenous NO production and that NO production is reduced in the absence of mitochondrial respiration. This latter finding, together with our recent findings that some YHb resides in the mitochondrial matrix (Cassanova et al., 2005), led us to ask if NO is produced in yeast mitochondria. We first asked if yeast mitochondria possess a NOS. Although several studies have reported the existence of a mitochondrial NOS (mtNOS) in mammals, there is currently no consensus concerning the identity of this protein (Brookes, 2004; Ghafourifar and Cadenas, 2005; Lacza et al., 2005). It has been characterized by various research groups as either neuronal (NOS1), inducible (NOS2), or endothelial (NOS3). To address whether yeast mitochondria have a NOS, we performed three types of analysis. First, we searched the yeast genome (www.yeastgenome.org) and yeast mitochondrial proteome for proteins related to any known NOS, using BLASTP. This analysis failed to reveal substantial sequence matches with homology to genes that encode the three mammalian NOS isoforms or the gene for AtNOS1, a putative NOS (Guo et al., 2003). Second, we subjected yeast mitochondria to immunoblot analysis using antibodies raised to the three mammalian NOS isoforms. From Figure S2 it is clear that we could not detect crossreacting material to any NOS isoform in yeast mitochondria. As expected, all three NOS isoforms are detected in the control, which consisted of cell extracts from embryonic mouse fibroblasts (Figure S2). Third, we assessed the ability of yeast mitochondrial lysates to produce NO by providing the NOS substrate (L-Arginine) and cofactors (NADPH, FAD, FMN, calmodulin, and calcium). This experiment also failed to reveal the presence of NOS in yeast mitochondria (data not shown). In contrast, we easily detected NOS activity in our positive rat brain control tissue. Together, these findings make it very unlikely that yeast mitochondria produce NO via a classical NOS.

NO_2^--dependent production of NO by yeast mitochondria

Our finding that the enhanced NO production seen in DR11 is not observed in DR10 (Figure S1) suggested a role for the mitochondrial respiratory chain in NO production. To address this we asked if yeast mitochondria produce NO when provided with either nitrate (NO_3) or NO_2^-. Given that YHb is located in mitochondria and is an effective consumer of NO we first used mitochondria from DR11. These were compared to mitochondria from JM43, which possesses wild-type YHb and a functional respiratory chain, and DR10, which lacks YHb and a respiratory chain. These studies revealed no NO production from NO_3 under any conditions. Initially, we also failed to detect NO production from NO_2^-, until we discovered that NO was produced only after a 10–12 min lag following introduction of mitochondria into the reaction chamber. In the experiment shown in Figure 1A, mitochondria were placed in NO assay medium and incubated for 12 min. Then NO_2^- was added to the reaction vessel and NO production followed. We could detect NO production by DR11

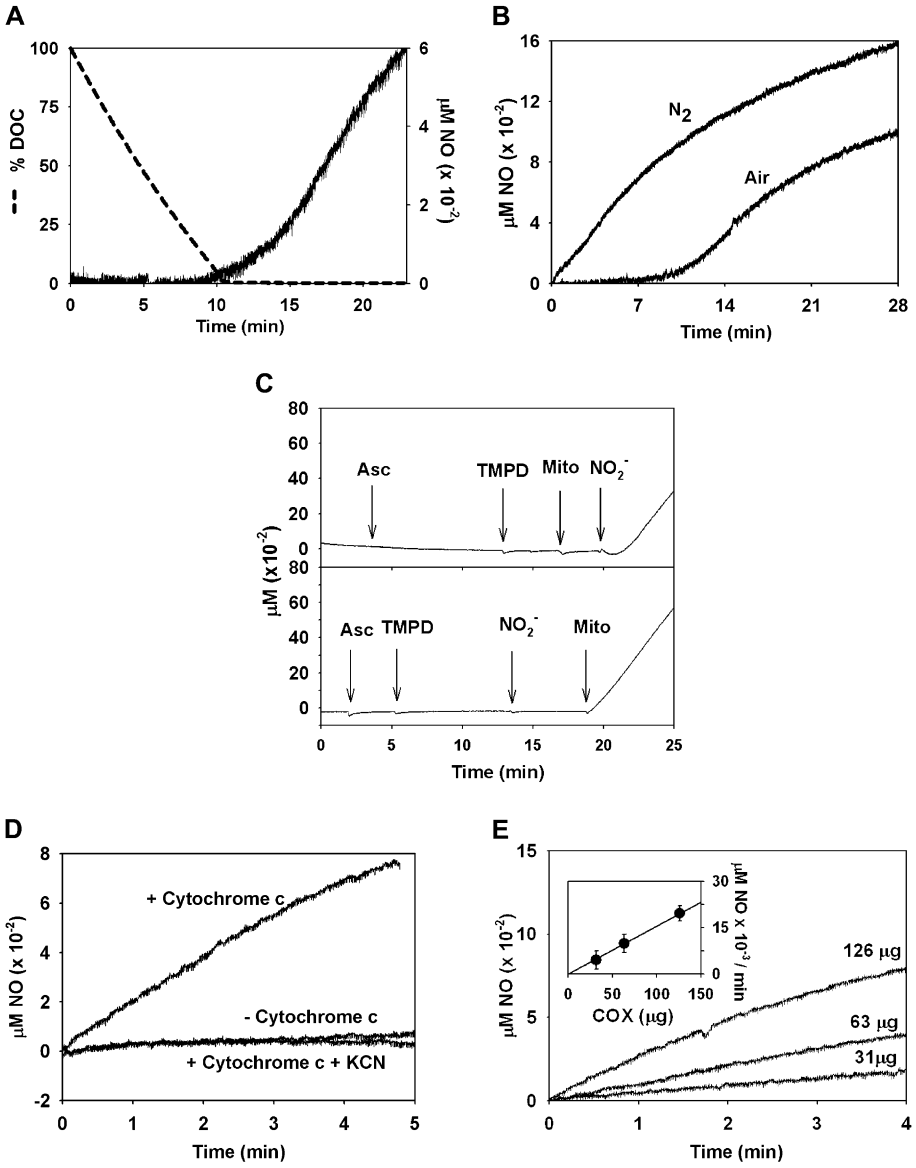

Figure 2. NO production by yeast mitochondria is inhibited by oxygen and catalyzed by cytochrome *c* oxidase

A) NO production by isolated DR11 mitochondria in the presence of different levels of dissolved oxygen. NO production from 400 μg protein/ml was measured as described in Figure 1A. Dissolved oxygen concentration was measured concomitantly with a Strathkelvin oxygen electrode. DOC = dissolved oxygen concentration.

B) NO production by isolated DR11 mitochondria in normoxic and anoxic conditions. NO production was measured as described in Figure 1D except that the assay buffer was pre-bubbled with either air or nitrogen, as indicated.

C) Ascorbate/TMPD supports NO_2^--dependent NO synthesis only in the presence of mitochondria. NO production was measured with an NO electrode in the assay medium (minus succinate) described in Figure 1A. At the time indicated by arrows, TMPD (0.5 mM final concentration), ascorbate (0.5 mM final concentration), 400 μg/ml mitochondria, or $NaNO_2$ (1mM final concentration) were added to the reaction chamber.

D) Effects of cytochrome *c* and KCN on cytochrome *c* oxidase catalyzed NO_2^--dependent NO production. One hundred and twenty-six micrograms of purified cytochrome *c* oxidase were added to the NO electrode chamber pre-bubbled with nitrogen for 5 min at 28°C in a medium containing: 650 mM Mannitol, 10 mM K_2HPO_4 (pH 6.5), 0.1 mM EDTA, 10 mM KCl, 2 μM cytochrome *c*, 1.25 mM TMPD, and 0.01% Triton X 100. $NaNO_2$ was added to the chamber (1 mM final concentration), and NO production was followed with NO electrode.

E) Dose-dependent NO synthesis by purified cytochrome *c* oxidase. Aliquots containing different amounts of purified cytochrome *c* oxidase were assayed for NO_2^--dependent NO synthesis production, as described above in (**D**). *Inset*, Initial rate of NO production versus μg cytochrome *c* oxidase protein.

mitochondria but not by mitochondria from JM43 or DR10. This suggests that the respiratory chain is required for NO production and that mitochondrial YHb is effective in consuming the NO that is produced in JM43 mitochondria. From the results shown in Figure 1B it is clear that the rate of NO production is dependent on the amount of mitochondrial protein assayed. As a control for NO production we used the NO scavenger PTIO (2-Phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl), which immediately reduced the level of NO produced under our assay conditions (Figure 1C). KCN inhibited continued NO production, supporting the conclusion that NO production requires a functional respiratory chain.

To determine the relationships between NO_2^- concentration and NO production, we performed assays at different external NO_2^- concentrations (Figure 1D). We also measured the amount of external NO_2^- that is taken up by mitochondria. The latter revealed that only 10% of the external NO_2^- is internalized. Results from this analysis reveal that NO production occurs over a wide range of NO_2^- concentrations and can be detected with as little as 20 μM internalized NO_2^-, which is within the range of NO_2^- concentrations found in yeast cells (see below).

Oxygen effects on NO_2^--dependent NO production

Our finding that preincubation of DR11 mitochondria in a closed vessel precedes NO_2^--dependent NO production suggested that the preincubation period functions to reduce oxygen levels in the assay buffer. To address this we measured the oxygen concentration in the vessel during the preincubation period (Figure 2A) and found that NO production began when the oxygen concentration fell below a dissolved oxygen concentration of 2% (20 μM O_2 under our assay conditions) and was maximal when oxygen was absent. This finding implies that oxygen affects NO_2^--dependent NO formation in mitochondria. To further explore this finding we compared the length of the lag period for mitochondria that had been placed in assay buffer pre-bubbled with air or nitrogen. Pre-bubbling with air had little effect (Figure 2B) while pre-bubbling with nitrogen completely removed the lag in NO production, providing direct support for the

conclusion that NO_2^--dependent NO production in mitochondria is inhibited by oxygen.

Involvement of the respiratory chain

Having determined that the preincubation lag functions to reduce the oxygen concentration in the reaction vessel, we modified our assays to include a 5 min pre-bubbling with nitrogen prior to the addition of NO_2^-. Using these assay conditions, we were able to establish that NO production by yeast mitochondria requires an electron acceptor (NO_2^- but not NO_3) and an electron donor (succinate, malate, pyruvate) (Table 1). This NO_2^--dependent NO production is inhibited by cyanide and antimycin A (Table 1) and is not present in DR10, which lacks YHb and is respiration deficient (Figure 1A). The finding that KCN prevents NO production when added at the beginning of the experiment (Table 1) and abruptly inhibits NO production when added later (Figure 1C) indicates that the respiratory chain is involved in NO production throughout the entire course of the experiment.

We have also found that the nonphysiological electron donors ascorbate/TMPD support a high level of NO production (Table 1) both in the presence and absence of antimycin A, a complex III inhibitor. From Figure 2C it is clear that the NO synthesis supported by ascorbate/TMPD requires mitochondria because there is no NO synthesis in their absence. This indicates that the high rates of NO production supported by ascorbate/TMPD are enzymatic and are not attributable to the nonenzymatic reduction of NO_2^- to NO. While the TCA cycle intermediates succinate, malate, or pyruvate feed electrons into the beginning of the respiratory chain, the electron donor pair ascorbate/TMPD feeds electrons into cytochrome *c* and then cytochrome *c* oxidase at the end of the chain. This finding, together with our observation that ascorbate/TMPD supports NO production under conditions where complex III is inhibited, clearly suggests that the terminal region of the respiratory chain is sufficient for NO synthesis.

NO production in intact yeast mitochondria is also affected by ADP (Table 1). There are at least two plausible explanations for this. First, ADP may increase the rate of NO production simply by dissipating the proton gradient across the inner mitochondrial membrane. This would result in an increased rate of electron flow through the respiratory chain. Alternatively, ADP could be converted to ATP, which may itself affect NO production. We addressed the first possibility by substituting the uncoupler dinitrophenol for ADP and the second possibility by substituting ATP for ADP. From Table 1 it is clear that ADP can be replaced by either dinitrophenol or ATP but not GTP or GDP. These results suggest that ADP increases the rate of NO production both by increasing the rate of electron transport and by its conversion to ATP. Finally, it is important to note that the NOS inhibitor, γ-nitro-L-Arginine-Methyl Ester (L-NAME), has no effect on NO_2^--dependent NO production in these mitochondria (Table 1). This rules out a role for NOS in NO_2^--dependent NO production.

Cytochrome *c* oxidase catalyzes NO_2^--dependent NO production

The finding that added ascorbate/TMPD supports a high rate of NO_2^--dependent NO formation in the presence of antimycin A suggested that cytochrome *c* and cytochrome *c* oxidase alone can function in reducing NO_2^- to NO in hypoxic/anoxic yeast mitochondria. To test this directly we asked if purified yeast cytochrome *c* oxidase has NO_2^- reductase activity, when assayed in the absence of oxygen. Isolated yeast cytochrome *c* oxidase, in assay medium containing cytochrome *c* and TMPD, was placed in the NO electrode reaction chamber and bubbled with nitrogen for 5 min to remove oxygen. NO_2^- was then added and NO production was measured. Cytochrome *c* and cytochrome *c* oxidase were added in equimolar amounts and TMPD was added in excess in order to keep the cytochrome *c* that is in the reaction buffer or bound to cytochrome *c* oxidase reduced (Cooper, 1990). It is clear from Figure 2D that isolated cytochrome *c* oxidase supports a robust rate of NO production and that its ability to produce NO is dependent on cytochrome *c* and is inhibited by cyanide, and air (data not shown). The rate of NO production by cytochrome *c* oxidase increases with increasing concentrations of enzyme (Figure 2E). In addition, this reaction is affected by pH (Table 2). Using buffers with pH values that correspond to the intracellular pH of yeast cells grown in the presence or absence of air (Campbellburk et al., 1987; Gonzalez et al., 2000; Valli et al., 2005), we find that NO production by cytochrome *c* oxidase increases between pH 7 and pH 6. We also find that we are better

Table 1. Characterization of yeast mitochondrial NO_2^--dependent NO production

Assay medium	Rate of NO production (%)[a]
NO Assay Medium[b]	100 ± 8
—NO_2^-	0 ± 5
—NO_2^- + 1mM NO_3^-	0 ± 4
—Mitochondria	0 ± 3
—Succinate	0 ± 5
+ 10 mM L-NAME	100 ± 7
—Succinate, + 2.5 mM Malate/pyruvate	210 ± 11
—Succinate + 5 mM NADH	0 ± 5
+ 1 mM KCN	0 ±4
+ 20 μg/ml Antimycin A	0 ± 5
—Succinate, + 0.5 mM ascorbate/TMPD	820 ± 30
—Succinate, + 0.5 mM ascorbate/TMPD + 20 μg/ml Antimycin A	790 ± 25
—ADP	12 ± 2
—ADP + 0.03 mM ATP	94 ± 2
—ADP + 0.03 mM GDP	2 ± 1
—ADP + 0.03 mM GTP	3 ± 1
—ADP + 0.2 mM Dinitrophenol	137 ± 5

NO production by intact yeast mitochondria (0.7 mg of mitochondrial protein) was determined with an NO electrode using a closed chamber in NO Assay Medium, as described in Experimental Procedures. Mitochondria were preincubated in the closed chamber for 12 min at 28ºC, prior the addition of $NaNO_2$. Values are given ± standard error of mean.

[a] 100% is equivalent to12 μM NO × 10^{-3}/min.

[b] NO Assay Medium: 30 μM ADP, 6 mM succinate, 650 mM Mannitol, 10 mM K_2HPO_4 (pH 6.5), 0.1 mM EDTA and 10 mM KCl.

Table 2. Effect of NO_2^- concentration and pH on NO synthesis by purified yeast COX

	NO Rate (μM × 10^{-3} /min)		
NO_2^- concentration (mM)	pH 6	pH 6.5	pH 7
0.02	1.3 ± 0.7	—	—
0.1	14 ± 0.8	1.1 ± 0.6	—
1	221 ± 0.9	17 ± 0.9	1.0 ± 0.5

Assays of yeast cytochrome *c* oxidase (63 μg of protein) were performed in 650 mM Mannitol, 10 mM K_2HPO_4, 0.1 mM EDTA, 10 mM KCl, 2 μM cytochrome *c*, 1.25 mM TMPD, and 0.01% Triton X 100, pre-bubbled with nitrogen for 5 min at 28ºC. $NaNO_2$ was then added (final concentration 1 mM) and NO synthesis measured in a closed chamber as described in Experimental Procedures. Values are ± SEM.

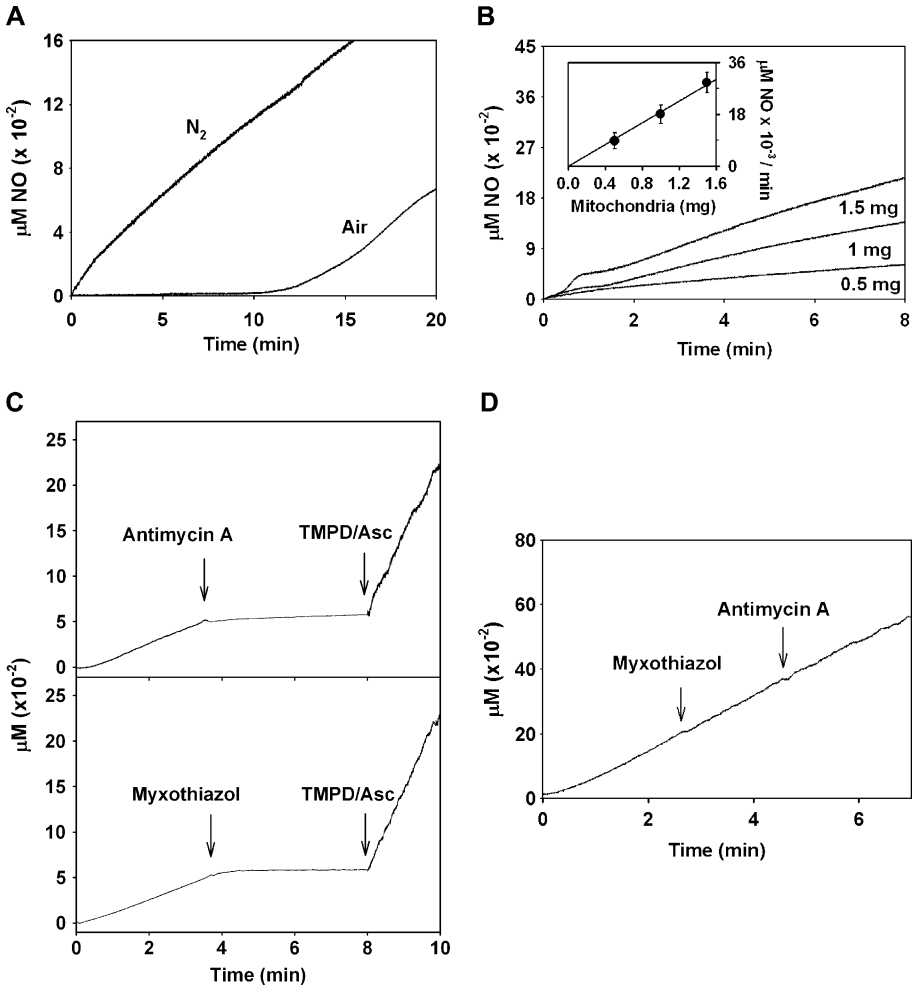

Figure 3. NO production by rat liver mitochondria

A) NO_2^--dependent NO synthesis by rat liver mitochondria in normoxic or anoxic conditions. NO production was measured as described in Figure 1A, using isolated rat liver mitochondria (400 μg protein/ml), prebubbled with either air or N_2, as indicated.

B) Dose-dependent NO synthesis in isolated liver mitochondria. Aliquots containing different amounts of liver mitochondrial protein were pre-bubbled with nitrogen for 5 min at 28ºC in NO Assay Medium. $NaNO_2$ was then added to a final concentration of 1 mM and NO production followed. *Inset*, Initial rate of NO production versus mg mitochondrial protein.

C) Effect of the inhibitors of complex III and TMPD on the production of NO by rat liver mitochondria. NO production was measured as described in (**A**) above, using isolated rat liver mitochondria (400 μg protein/ml). At the times indicated by arrows, Antimycin A, Myxothiazol, or acorbate/TMPD were added to final concentrations of 20 μg/ml, 4 μM, or 0.5 mM/0.5 mM, respectively.

D) TMPD-dependent activity is not affected by the inhibitors of complex III. Rat liver mitochondria (400 μg protein/ml) were pre-bubbled with nitrogen at 28ºC for 5 min in NO Assay Medium (minus succinate, plus 0.5 mM/0.5mM ascorbate/TMPD). $NaNO_2$ was then added to a final concentration of 1 mM and NO production followed with a NO electrode. At the times indicated with arrows, Antimycin A and Myxothiazol were added to final concentrations of 20 μg/ml and 4 μM, respectively.

able to measure this activity with lower NO_2^- concentrations at pH 6.0 than at a pH of 6.5 or 7.0. This finding is interesting for two reasons. First, because the intracellular pH of yeast and other cells drops when cells are exposed to anoxia or hypoxia. This finding suggests that the ability of cytochrome *c* oxidase to function as a NO_2^- reductase in anoxic cells or mitochondria is mediated by changes in intracellular pH. And second, it is well accepted that the rate of electron transport from heme a to heme a_3 in the binuclear reaction center of cytochrome *c* oxidase, with oxygen as substrate, is proton coupled. It increases with decreasing pH because this enzyme also functions as a proton pump when present in the inner mitochondrial membrane (Cooper, 1990). Our findings suggest that this also occurs when NO_2^- is the electron acceptor.

NO_2^--dependent NO production by rat liver mitochondria

To examine whether mitochondria from higher organisms exhibit NO_2^--dependent NO production under the conditions used for yeast mitochondria, we assayed isolated rat liver mitochondria. As with yeast mitochondria, a lag is observed prior to NO synthesis unless the assay buffer is pre-bubbled with nitrogen (Figure 3A). The rate of NO synthesis increases in a dose-dependent fashion with increasing mitochondrial protein (Figure 3B). This activity, like that in yeast mitochondria, requires NO_2^- (but not nitrate), an electron donor, and ADP. It can be supported either by physiological electron donors (succinate, malate, pyruvate, NADH) or by ascorbate/TMPD (Table 3) and is inhibited by antimycin A, myxothiazol, KCN, and carbon monoxide. NO_2^--dependent NO synthesis in rat liver mitochondria is supported by wide-range external NO_2^- concentrations and can be observed with as little as 100 μM added NO_2^- (data not shown). As with yeast mitochondria only 10% of the added NO_2^- is internalized by rat liver mitochondria. NO_2^--dependent NO synthesis in rat liver mitochondria is not inhibited by L-NAME, which supports the conclusion that none of the NO synthesis observed is attributable to a mtNOS. This is not surprising because our assay conditions lack L-Arginine and calcium and have a pH that is too acidic for NOS activity (Conte, 2003).

Our results confirm those of a previous study, which also reported that rat liver mitochondria are capable of recycling NO_2^- to NO under anoxic conditions (Nohl et al., 2000). In contrast to our findings above with yeast mitochondria, this later study concluded that complex III of the respiratory chain functioned as the NO_2^- reductase. To examine this possibility, we looked at the effects of the two complex III inhibitors, antimycin and myxothiazol, on NO production in rat liver mitochondria, provided with either succinate or TMPD as a source of electrons. Both inhibitors prevent the production of NO from NO_2^- when succinate is used as a source of electrons but not when TMPD is used as a source of electrons (Figure 3C). However,

Table 3. Characterization of rat liver mitochondrial NO_2^--dependent NO production

Assay medium	Rate of NO production (%)[a]
NO Assay Medium[b]	100 ± 8
—NO_2^-	0 ± 9
—NO_2^- + 1 mM NO_3^-	0 ± 8
—Mitochondria	0 ± 7
—Succinate	0 ± 6
+10 mM L-NAME	100 ± 9
—Succinate, + 2.5 mM Malate/pyruvate	260 ±13
—Succinate + 5 mM NADH	0 ± 6
—ADP	50 ± 6
—Succinate + 0.5 mM ascorbate/TMPD	700 ± 80
+ CO (bubbled 10 min)	0 ± 7
+ 1 mM KCN	0 ± 5
—Succinate + 0.5 mM ascorbate/TMPD + CO	2 ± 5
—Succinate + 0.5 mM ascorbate/TMPD + 1 mM KCN	3 ± 5
—Succinate + 20 μg/ml Antimycin A	15 ± 6
—Succinate + 4 μM Myxothiazol	0 ± 5
—Succinate + 0.5 mM ascorbate/TMPD + 20 μg/ml Antimycin A	650 ± 70
—Succinate + 0.5 mM ascorbate/TMPD + 4 μM Myxothiazol	680 ± 50

NO production by intact rat liver mitochondria (0.7 mg of mitochondria protein) was determined with an NO electrode using a closed chamber in NO Assay Medium, as described in Experimental Procedures. Mitochondria were preincubated in the closed chamber for 12 min at 28°C prior the addition of $NaNO_2$. Values are given ± standard error of mean.

[a] 100 % is equivalent to12 μM NO × 10^{-3}/min.

[b] NO Assay Medium is 0.7 mg of mitochondria, 30 μM ADP, 6 mM succinate, 650 mM Mannitol, 10 mM K_2HPO_4 (pH 6.5), 0.1 mM EDTA, and 10 mM KCl.

ascorbate/TMPD supports the resumption of NO_2^--dependent NO synthesis, after blocking succinate-driven electron transport with either inhibitor. In addition, neither myxothiazol nor antimycin A inhibit TMPD-driven NO production (Figure 3D). Because TMPD feeds electrons into the respiratory chain downstream of complex III (at the level of cytochrome *c*), these data support the conclusion that the terminal portion of the respiratory chain (i.e., cytochrome *c* and cytochrome *c* oxidase) is sufficient for the reduction of NO_2^- to NO in rat liver mitochondria, as we have found in yeast mitochondria. This conclusion is further substantiated by our finding that carbon monoxide, which specifically inhibits cytochrome *c* oxidase, blocks the reduction of NO_2^- to NO in rat liver mitochondria (Table 3).

NO affects expression of CYC7

Previously, we have demonstrated that induction of the hypoxic nuclear gene *CYC7* in yeast cells requires cytochrome *c* oxidase and the respiratory chain (Kwast et al., 1999). We have also shown that cyanide blocks the induction of *CYC7* after cells are anoxic (Kwast et al., 1999). This suggested that cytochrome *c* oxidase functions with an alternative electron acceptor in the absence of oxygen and that the product of this reaction functions as a "signal" that is required for the induction of *CYC7*. Given the above finding that cytochrome *c* oxidase can use NO_2^- as an alternative electron acceptor to produce NO under anoxic conditions we next asked if NO is a "signal" involved in the hypoxic induction of this gene. To address this we performed three types of experiments. First, we asked if YHb influences the kinetics of induction of *CYC7*. From Figure 4A it can be seen that the expression of *CYC7* in JM43 does not increase until 8 hr after the shift. In contrast, expression of *CYC7* in DR11 increases between 2 and 4 hr after a shift, reaching a maximum at 8 hr, and then declines. This clearly demonstrates that the kinetics of induction of this gene are different in JM43 and DR11. Second, we compared the level of expression of *CYC7* in JM43, DR11, and DR10 cells grown to steady state under anoxic conditions. The level of *CYC7* expressed during steady-state anoxic growth is much higher in DR11 than it is in either JM43 or DR10 (Figure 4B). Together, these findings indicate that the absence of YHb affects both the kinetics of hypoxic induction and the level of expression of *CYC7* in anoxic cells. They also suggest that the enhanced level of *CYC7* expression seen in DR11 requires both the absence of YHb and the presence of a functional respiratory chain. The simplest explanation for these observations is that the enhanced level of nitrosative stress experienced by cells lacking YHb influences the expression of *CYC7*. To examine this more directly, we asked if an exogenously supplied NO donor, diethylenetriamine NO adduct (DETA-NO), affects the expression of *CYC7* when added to aerobic DR11 cells. This experiment revealed that low concentrations of DETA-NO increase the expression of *CYC7.* Normoxic cells treated with 0.5 mM DETA-NO show a 7-fold increase in CYC7 expression (Figure 4C). When considered together, these findings indicate that NO plays a role in the expression of *CYC7* and suggest that mitochondrially generated NO functions early in a hypoxic signaling pathway.

Protein tyrosine nitration increases in hypoxic cells

The above studies have focused primarily on DR11, a strain that is *null* for YHb. In order to ask if YHb^+ cells experience nitrosative stress when exposed to anoxia we subjected JM43 cells to a shift from normoxia to anoxia and examined the levels of protein tyrosine nitration at different times after the shift. From Figure 4D it is obvious that the level of protein tyrosine nitration of specific protein bands increases immediately after the shift and reaches a peak at 3 hr. Interestingly, this transient increase in protein tyrosine nitration parallels the transient increase in oxidative stress, measured as protein carbonylation (Dirmeier et al., 2002); both reach a peak at 3 hr after the shift. Because peroxy NO_2^- functions in protein tyrosine nitration and is formed from superoxide and NO, these findings strongly suggest that levels of both superoxide and NO increase upon exposing these cells to anoxia. Given that YHb in JM43 is present in both the mitochondrial matrix and cytosol (Cassanova et al., 2005), these findings also imply that the level of mitochondrial YHb present at the time of the shift is insufficient to counter NO production when the respiratory chain goes anoxic. Moreover, the ability of YHb to consume NO in both the presence or absence of oxygen (Liu et al., 2000) and to localize between the mitochondrial matrix and cytosol in an oxygen-dependent manner (Cassanova et al., 2005) strongly suggests that YHb functions to coordinate intracellular NO levels with environmental oxygen levels.

Discussion

Mitochondrial NO synthesis

The results presented here indicate that mitochondria from yeast and rat liver are capable of NO_2-dependent NO synthesis and that this occurs independently of a mtNOS. NO_2^--dependent NO synthesis requires the respiratory chain and appears when the oxygen concentration drops below ~20 μM. The

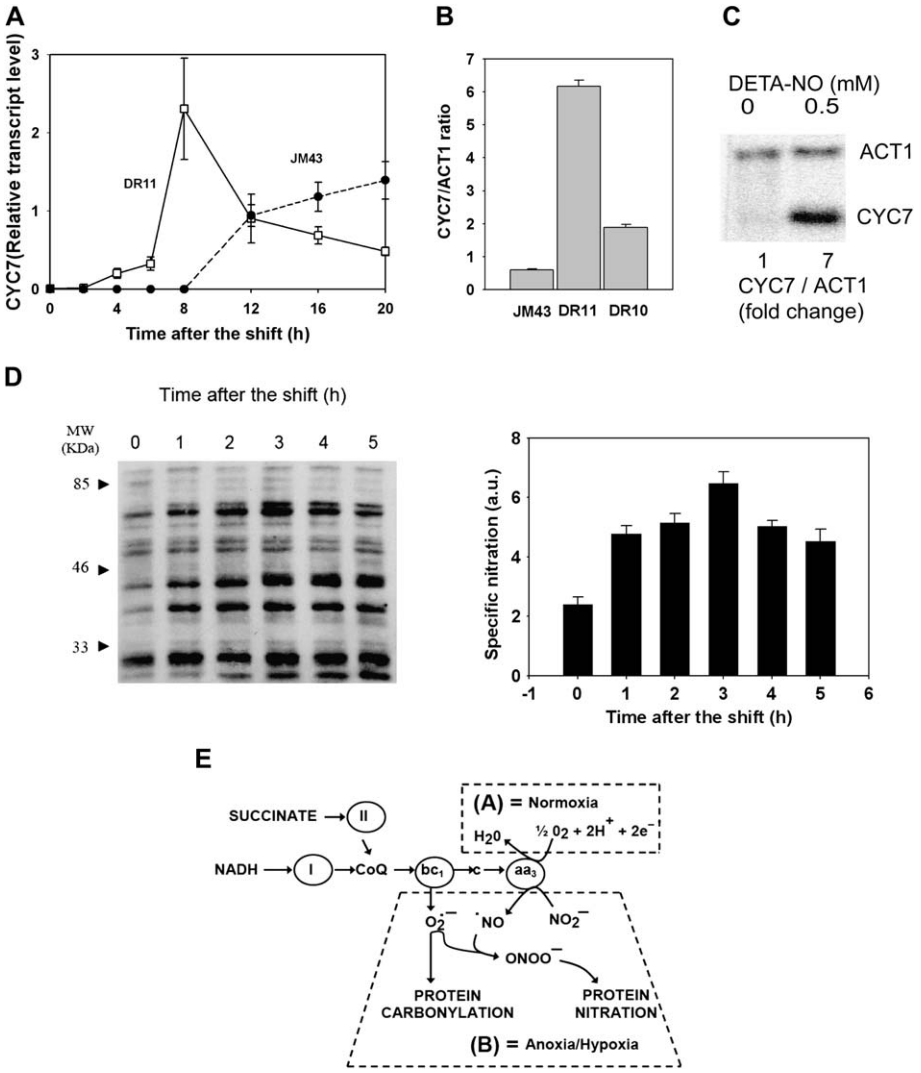

Figure 4. NO and the expression of CYC7 in *S. cerevisiae*

A) Effects of YHb on the hypoxic induction of *CYC7*. JM43 and DR11 cells were maintained in steady-state normoxic growth in the fermentor for six generations prior to changing the sparge gas from air to 97.5% N_2, 2.5% CO_2. Cells were harvested at the times indicated, their RNA extracted, separated by agarose electrophoresis, blotted, and hybridized with a *CYC7* probe. The transcript levels are expressed relative to the level at 12 hr. Values are ± SEM.

B) Expression of *CYC7* in cells grown to steady state under anoxic conditions. JM43, DR11, and DR10 were grown in 97.5% N_2, 2.5% CO_2 in the fermentor for six generations, harvested, and analyzed for the expression of the *CYC7* and *ACT1*. Values are ± SEM.

C) Induction of *CYC7* by DETA-NO. Expression of *CYC7* and *ACT1* in the presence of the NO donor, DETA-NO. DR11 yeast cell were examined and quantified by Northern blot hybridization as indicated in Experimental Procedures.

D) Protein tyrosine nitration in whole-cell extracts from JM43 after a shift to anoxia. *Left Panel*, JM43 cells were grown in the conditions indicated in (**A**). Cells were harvested at the times indicated, and 5 μg of protein of the whole-cell lysate were prepared and analyzed by SDS-PAGE electrophoresis and immunoblotting as described in Experimental Procedures. *Right Panel*, quantification of protein tyrosine nitration after a shift to anoxia. Values are ± SEM.

E) A model for the role of cytochrome *c* oxidase and the mitochondrial respiratory chain in hypoxic gene induction. In the presence of air, cytochrome *c* oxidase uses oxygen as an electron acceptor and produces water. When oxygen is limiting, it uses NO_2^- as an electron acceptor and produces NO. Under low-oxygen conditions, the respiratory chain becomes reduced and the electrons that accumulate are released as superoxide ($O_2^{\cdot-}$) by cytochrome bc_1. This superoxide combines with NO to produce peroxynitrite ($ONOO^-$), which promotes protein tyrosine nitration of specific proteins that may be involved in a signaling pathway to the nucleus.

NO_2^--dependent NO synthesis that we observe is catalyzed by cytochrome *c* oxidase and is observable at effective internal mitochondrial NO_2^- concentrations as low as 20 μM, which is within the physiological range. These findings are interesting in the context of several other studies that have addressed the effects of hypoxia on mitochondrial NO synthesis. For example, it has been reported that NO production in rat liver and heart mitochondria increases under hypoxic conditions (Valdez et al., 2004; Schild et al., 2003) and that some of this activity is not inhibited by the NOS inhibitor L-NAME. In addition, a NOS-independent pathway in ischemic heart has been linked to NO_2^--dependent NO production (Tiravanti et al., 2004). This latter finding is especially interesting because nitroglycerin, which is used to treat ischemia, is metabolized to NO_2^- and then NO under hypoxic conditions (Agvald et al., 2002), and because NO_2^- is produced from nitroglycerin in mitochondria (Chen et al., 2002). Finally, mitochondria from a variety of eukaryotes are capable of reducing NO_2^- to NO when incubated at low-oxygen concentrations (Kozlov et al., 1999; Nohl et al., 2000; Planchet et al., 2005; Tiravanti et al., 2004; Tischner et al., 2004). This activity is not attributable to mtNOS.

Cytochrome *c* oxidase as a NO_2^- reductase

It has been reported that under some conditions, mammalian cytochrome *c* oxidase can convert NO_2^- to NO as a NO_2^- reductase (Paitian et al., 1985) and that a heme a_3–nitrosyl complex is formed at the binuclear reaction center in bovine heart cytochrome *c* oxidase when incubated in the presence of excess NO_2^- and a reducing agent (Brudvig et al., 1980). At pH 7.3, NO dissociates from this complex with a rate of $0.01 s^{-1}$ (Sarti et al., 2000), indicating that under these conditions bovine heart cytochrome *c* oxidase can function as a slow NO_2^- reductase. Here, we have shown that purified yeast cytochrome *c* oxidase can also function as a NO_2^- reductase. It does so when oxygen is limiting and the rate of the reductase reaction increases with decreasing pH. This is interesting because the intracellular pH of yeast cells decreases when cells are exposed to anoxia (Campbellburk et al., 1987; Gonzalez et al., 2000). Yeast cytochrome *c* oxidase can produce NO over a wide range of NO_2^- concentrations. We have determined that the NO_2^- concentration in aerobic JM43 yeast cells ranges from 9.5 to 17.5 nmol of NO_2^- per g wet weight. Assuming that one g wet weight is equivalent to 0.64 ml cell water (Sherman, 2002),

this value corresponds to 15 to 27.5 μM NO_2^-. This range of NO_2^- concentrations partially overlaps the range of NO_2^- concentrations used by yeast cytochrome *c* oxidase and yeast mitochondria.

Mitochondrially produced NO and hypoxic gene induction

Although several previous studies have implicated the respiratory chain in the induction of hypoxic nuclear genes in both yeast (Kwast et al., 1999; Poyton, 1999) and mammals (Poyton, 1999; Poyton et al., 2003; Chandel et al., 1998) the molecular mechanisms that underlie this are unclear. One possibility is that the respiratory chain produces increased levels of ROS when cells are exposed to anoxia or hypoxia and that these ROS are involved (Figure 4E). Although this mechanism for hypoxic gene induction in mammals has been controversial in the past (c.f., Agani et al., 2000; Chandel et al., 1998, 2000; Enomoto et al., 2002; Srinivas et al., 1999; Srinivas et al., 2001; Vaux et al., 2001), recent studies strengthen the conclusion that mammalian cells undergo oxidative stress when exposed to hypoxia and that mitochondrially generated ROS function in hypoxic signaling by stabilizing HIF-1α (Brunelle et al., 2005; Guzy et al., 2005; Mansfield et al., 2005). However, it is not clear from these studies that ROS are sufficient for HIF-1α stability. Indeed, it is unlikely that mitochondrially generated ROS are sufficient for the induction of hypoxic genes in yeast because exogenous oxidants do not induce their expression (Causton et al., 2001; Gasch et al., 2000). This suggests that the respiratory chain participates in hypoxic signaling in other ways.

Our findings that mitochondria produce NO under hypoxic or anoxic conditions and that the level of protein tyrosine nitration in yeast cells is increased in a protein-specific manner when cells are shifted from normoxia to anoxia provide an alternative model for mitochondrial participation in hypoxic signaling. Indeed, they suggest a model (Figure 4E) in which mitochondrially generated NO and superoxide combine to form peroxynitrite, which then tyrosine nitrates protein components of a hypoxic signaling pathway. Support for this mitochondrial model for hypoxic signaling in yeast comes from our finding that NO induces the expression of yeast *CYC7*, a hypoxic nuclear gene, that *CYC7* is induced earlier and at higher levels in *yhb1*$^-$ cells than in *YHB1*$^+$ cells, and that *YHB1*$^+$ cells undergo a transient increase in tyrosine protein nitration upon exposure to reduced oxygen levels. This model also receives support from recent mammalian cells studies, which have shown that cytochrome *c* is required for the stabilization of HIF-1α in murine cell lines exposed to hypoxia (Mansfield et al., 2005). Cytochrome *c* is a substrate for cytochrome *c* oxidase and hence required for NO_2-dependent NO production. However, this model is confounded by previous attempts to evaluate the involvement of NO in mammalian hypoxic signaling because these studies have produced contradictory results. Some have reported that NO inhibits hypoxia-induced HIF-1α stabilization (Hagen et al., 2003; Huang et al., 1999; Liu et al., 1998; Sogawa et al., 1998) while others have reported that NO serves to stabilize HIF-1α in normoxic cells (Kimura et al., 2002; Metzen et al., 2003). It has also been reported that NO and other respiratory inhibitors lead to increased intracellular oxygen levels in hypoxic cells and that this increase in intracellular oxygen levels suppresses HIF-1α stabilization (Hagen et al., 2003), a conclusion that was challenged recently (Brunelle et al., 2005). It is not yet known if peroxynitrite affects the stability of HIF-1α, possibly via protein tyrosine nitration. In this regard, it is useful to note that superoxide and peroxynitrite are capable of affecting protein activity/stability under conditions where NO alone has no effect (Hausladen and Fridovich, 1994).

Clearly, more work is required to test the validity of the above model. Attempts to examine the role of mitochondrially produced NO and peroxynitrite as well as protein tyrosine nitration in hypoxic signaling in yeast and mammalian cells are currently underway.

Experimental procedures

Yeast strains and growth media

The following yeast strains of *S. cerevisiae* were used: JM43 (Mat α *his4-580 trp1-289 leu 2-3, 112 ura3-52* [ρ$^+$]) (McEwen et al., 1986); DR11, a derivative of JM43 containing a *URA3* disrupted *YHB1* gene; and DR10, a derviative of JM43 ρ^0 (Mat α *his4-580 trp1-289 leu 2-3, 112 ura3-52* [ρ^0]) containing a *URA3* disrupted *YHB1* gene (Zhao, et al., 1996). Strains JM43, DR10, and DR11 are isochromosomal except for the *yhb null* mutations carried in DR10 and DR11. JM43 cells were grown in SSG-TEA media (supplemented with Tween 80, ergosterol, amino acids, and uracil, as needed [Kwast et al., 1999]). DR11 and DR10 cells were grown in SSG-TE (URA-) dropout media (per liter: 3 g Difco Yeast Nitrogen Base, w/o amino acids, 10 g galactose, 0.8 g NH_4SO_4, 1 g KH_2PO_4, 0.5 g NaCl, 0.34 g $MgSO_4$, 5 μg $FeCl_2$, 2 g amino acid dropout mix (-ura) and 0.4 g $CaCl_2$) supplemented with 0.1% Tween 80 and 20 μg/ml ergosterol. Aerobic cultures and pre-cultures were grown in a shaker (200 rpm) at 28ºC and harvested in logarithmic growth phase. For shift experiments between normoxia and anoxia, a New Brunswick BioFlo 3000 fermentor was used as described (Poyton et al., 2004).

Preparation of whole-cell lysate, mitochondrial, and cytosolic fractions

Coupled mitochondria (i.e., with P:O ratios near 2) were prepared from aerobic cultures as described (McKee and Poyton, 1984). After cell breakage and centrifugation at 2000 × g for 3 min, an aliquot of the supernatant was saved as "whole-cell lysate." The rest of the supernatant was decanted and centrifuged for 10 min at 12000 × g to pellet the mitochondrial fraction, saving the supernatant as the cytosolic fraction.

Isolation of rat liver mitochondria

Rat liver mitochondria were prepared from 3-month-old F344/NHSD rats according to a published procedure (Pallotti and Lenaz, 2001).

Measurement of NO production

NO production was measured with a Clark-type NO electrode and an ISO-NO mark II NO-meter (WPI, Sarasota, Florida). Except where noted all solutions were NO_2^- free. Measurements were performed at 28ºC using a final reaction volume of 2 ml in a thermostated chamber with a close-fitting lid and fine holes for the electrode and a Hamilton syringe. Assays for NO production by the mitochondrial respiratory chain or cytochrome *c* oxidase were performed in NO Assay Medium (30 μM ADP, 6 mM succinate, 650 mM Mannitol, 10 mM K_2HPO_4 [pH 6.5], 0.1 mM EDTA, and 10 mM KCl).

SDS-PAGE

Mitochondrial and cytosolic fractions were separated on 10% polyacrylamide gels (*resolving gel*: 10% (w/v) 32:1 acrylamide: bisacrylamide, 0.1% (w/v) SDS, 0.4 M Tris, pH 8.8; *stacking gel*: 3.5% (w/v) 32:1 acrylamide: bisacrylamide, 0.1% (w/v) SDS, 0.125 M Tris, pH 6.8. The cytosol and mitochondrial protein samples were prepared by adding 2× SDS loading buffer (0.02 M $NaPO_4$, pH 6.8, 4% recrystallized SDS, 40 mM dithiothreitol, 8% glycerol) and then boiled for 3 min. The gel was run at 110 V until the dye front reached the bottom of the gel.

Northern blot analysis

RNA isolation, electrophoresis, Northern blotting, and hybridization were performed as indicated elsewhere (Poyton et al., 2004). The resulting images were analyzed and quantified using ImageQuant 5.2 (Molecular Dynamics).

Measurement of mitochondrial respiration

Rates of oxygen uptake by mitochondria were measured at 30ºC with a Strathkelvin oxygen electrode system, as described previously with some modifications (Poyton et al., 2004). The assay solution consisted of 2 ml of buffer containing 0.65 M Mannitol, 0.01M K_2HPO_4 (pH 6.5), 0.1 mM EDTA, 0.01 M KCl, and 0.7 mg of mitochondrial protein.

Measurement of intracellular NO_2^- concentration in normoxic cells

Aerobic cultures were grown on a shaker (200 rpm) at 28ºC and harvested in logarithmic growth phase. Cells were washed three times with cold distilled water, weighed, and suspended in 2 volumes of cold water. The cells were broken at 4ºC by sonication using a Branson Sonifier 250 with a microtip, a duty cycle of 20%, and a power setting of 40 W. The lysate was centrifuged 20 min at 14000 × g and an aliquot of the supernatant introduced via a Hamilton syringe in the chamber of the NO-electrode filled with a solution of 0.1 M H_2SO_4 and 0.1 M KI. The intracellular concentration of NO_2^- was calculated from the amperometric detection of NO produced from the NO_2^- present in the sample (Berkels et al., 2001).

Evaluation of NO_2^- uptake by mitochondria

Rat liver mitochondria or yeast mitochondria (1 mg) were incubated for 12 min at 28ºC in 2 ml of NO Assays Medium minus succinate and $NaNO_2$. After incubation, mitochondria were sedimented by centrifugation (13000 × g, 10 min) and their volume measured. The pellet was washed with 2 ml of 650 mM Mannitol, 10 mM K_2HPO_4 (pH 6.5), 0.1 mM EDTA, 10 mM KCl, 30 μM ADP and resuspended in 100 ul of 1% Triton X-100. NO_2^- concentration was measured both in the supernatant and in the pellet as indicated in the section above.

NO donor experiments

DR11 cells were grown in SSG-TE (URA-) drop out media as indicated above, harvested in logarithmic phase, washed twice with ice cold water, suspended in a medium containing 0.45% glucose, 40 mM K_2HPO_4 (pH 7.4) and incubated for 4 hr in a shaker (200 rpm) with different concentrations of DETA-NO. After incubation, the cells were harvested and mRNA extracted as indicated above.

Data analysis

All experimental data shown were derived from atleast two or three independent experiments.

Miscellaneous

Protein concentration was determined as described (Lowry et al., 1951), using bovine serum albumin as a standard. Cytochrome *c* oxidase from *S. cerevisiae* was prepared by Method 1 described previously (Poyton et al., 1995).

Supplemental data

Supplemental data include two figures and experimental procedures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/3/4/277/DC1/.

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